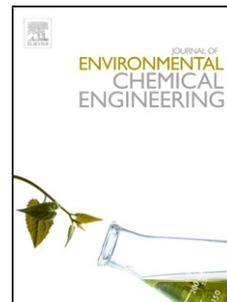


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Polycyclic aromatic hydrocarbons removal by immobilized bacterial cells using annonaceous acetogenins for biofilm formation stimulation on polyurethane foam

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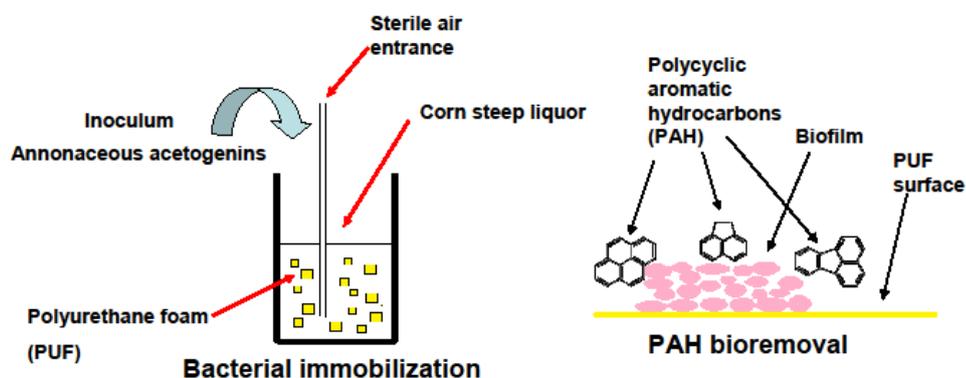
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Highlights:

- Biofilm formation on polyurethane foam was stimulated by annonaceous acetogenins.
- Almost 100% PAH removal was obtained with the different treatments assayed.
- PAH removal was achieved mostly by sorption phenomena.
- PAH bioremoval was about 20%.
- Biofilm formation stimulation did not correlate with improved PAH bioremoval.

Abstract

Wastewaters containing polycyclic aromatic hydrocarbons (PAH) must be treated before discharge in water bodies to avoid environmental pollution and comply with

environmental protection regulations. The development of novel PAH removal technologies from wastewaters is thus of great importance. The aim of this work was to use immobilized bacteria on polyurethane foam (PUF) for acenaphthene, fluoranthene and pyrene removal using annonaceous acetogenins (ACG) to stimulate biofilm and possibly enhance PAH removal activity. Different ACG were tested for their capacity to stimulate biofilm formation on *Pseudomonas monteilii* P26, a known naphthalene degrader. Itrabin, jetein and an ethanolic extract of *Annona cherimola* pulp were selected for showing a high stimulation level at low concentration in microplate biofilm formation assay. On PUF, the biofilm formation was strongly stimulated by itrabin and the ethanolic extract. However, there was no difference between the PAH removal percentages of the different systems (sterile PUF, cell immobilized in presence of ACG and cells immobilized without ACG). In average, 99% acenaphthene, 98% fluoranthene and 92.5% pyrene were removed in 7 days mostly by sorption (initial PAH concentration was 50 ppm). A 15-22% removal was attributed to biodegradation or bioaccumulation in the systems with immobilized cells. In this case, stimulating biofilm formation did not enhance PAH removal by immobilized bacteria on PUF.

Keywords: PAH; annonaceous acetogenins; biofilm; biodegradation; polyurethane foam.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are found in domestic, industrial wastewater and urban storm water [1,2,3]. They originate from incomplete combustion of fossil

fuels and are characterized for presenting two or more benzene fused rings. PAH are persistent pollutants and have carcinogenicity and mutagenicity properties [2]. Since these compounds are classified as priority pollutants by the majority of the environmental protection agencies of the world, the discharge of water that contains them into receptor water bodies is regulated. Therefore, maximum concentration limits of PAH in wastewater must be observed. To comply with regulations, economic and efficient wastewater treatment methods have to be developed as it is reflected in the extensive literature that has been published in recent years about the subject [4,5,6,7]. The use of bacteria immobilized in biofilm for PAH removal from a liquid phase could be a promising technology. It is well known that biofilms exhibit advantages over planktonic cells regarding its resilience against a harmful environment as could be a wastewater [8]. Besides, since the biofilm is attached to a surface, it could be easily removed from the liquid without using costly separation techniques. In addition, the biofilm support surface could contribute to the removal of contaminants if it possesses sorbent capacity. The addition of stressing factors during the cell immobilization process can stimulate biofilm formation increasing cell density and subsequent contaminant removal [9,10]. Annonaceus acetogenins (ACG) are natural stressors isolated from plants of the Annonaceae family [11,12]. They have cytotoxic activity and have been studied as antitumoral, antiparasitic and pesticidal compounds [13,14]. They have also been proposed as biofilm formation stimulants for a *Pseudomonas plecoglossicida* strain [11,15,16]. The aim of this work is to use ACG to produce high density biofilms of *Pseudomonas monteilii* P26 to be used in the removal of acenaphthene, fluoranthene and pyrene from water. These pollutants are, respectively, the numbers 001, 039 and 084 in the US EPA priority pollutant list [17]. This means that these pollutants are regulated by this agency and therefore their disposal limited.

The ACG itrabin, jetein, laherradurin and squamocin, as well as an ethanolic crude extract of *Annona cherimola* seeds, were tested for biofilm stimulation. Itrabin and jetein are two saturated γ -lactone which present 35 carbon atoms with two or one tetrahydrofuran (THF) rings respectively [18]. Laherradurin and squamocin are, respectively, saturated and unsaturated γ -lactone with 37 carbon atoms and two THF rings [16] (fig. 1). Polyurethane foam (PUF) was used as support for biofilm formation because of its low cost and known sorbent capacity of hydrophobic compounds [19]. Corn steep liquor was used as culture medium since it is a low cost industrial waste.

2. Materials and methods

2.1 Microorganisms and culture conditions

The strain *Pseudomonas monteilii* P26 (Genbank Acc. Num. HE798531) was isolated from sediments contaminated with petroleum oil originated from Patagonian coast, in Caleta Cordova, Chubut, Argentina [20]. Pure cultures were routinely maintained in JPP broth (% m/v: NaCl, 2; yeast extract, 0.1; meat peptone, 0.2; pH = 7.0) [21] with 20% v/v glycerol at -20 °C. Cell propagation was carried out in corn steep liquor (10% v/v, pH 7 and sterilized by heat at 121 °C for 15 min) at 30 °C and 180 rpm for 48 hours. The inocula were obtained from frozen stock cultures and its final concentration was 5% v/v.

2.2 Extraction, purification and characterization of annonaceous acetogenins

Itrabin, laherradurin, jetein and squamocin (ACG) were extracted by maceration in ethanol of dried and grinded *Annona cherimola* seeds (1000 g). Vacuum evaporation of

the solvent yielded an ethanolic extract. The ethanolic extract was first partitioned in a mixture of chloroform and water and then into hexane and methanol. The methanol sub-extract was obtained by evaporation and was subjected to separation on silica gel column using chloroform, ethyl acetate and methanol as eluants (gradient mode). ACG present in the column fractions were purified using RP-HPLC and mixtures of methanol/water as the elution solvent. A C18 (25 x 1 cm i.d., 10 µm particle size) column was employed. Fractions containing ACG were selected by TLC (Kedde reagent). The column fraction containing itrabin and laherradurin eluted with ethyl acetate: methanol 80:20 and its constituents were further purified with methanol: water 92:8. The column fraction containing jetein and squamocin was eluted with methanol and then purified with methanol: water 90:10. ACG were isolated as cereous white solids. Structural characterization and further identification was achieved by NMR (^1H , ^{13}C), EIMS and the comparison with previously reported data (for itrabin, laherradurin and jetein see [18] and [22] for squamocin).

An ethanolic extract of *Annona cherimola* pulp (1000 g) was also prepared by maceration in ethanol.

2.3 Biofilm formation screening

The effects of ACG and *Annona cherimola* pulp ethanolic extract on *P. monteilii* P26 biofilm formation on polystyrene microplates were evaluated by crystal violet staining and subsequent absorbance measurements at 560 nm [23]. Different concentrations of purified ACG (squamocin, laherradurin, jetein and itrabin: 1.25 and 2.5 ppm) and ethanolic extract of *Annona cherimola* pulp (extract: 0.25 and 0.025% w/v) were assessed. Ethanol was used as solvent and therefore was equally supplemented in

control assays (1% v/v). *P. monteilii* P26 inocula were prepared as explained in previous section and supplemented (10% v/v) into each well. Corn steep liquor 10 % v/v culture medium was employed. Microtiter plates were incubated for 6 h (28 °C) and results were recorded on a spectrophotometer (Biotek- Power Wave XS2 with GEN5 data analysis software). ACG which presented the highest biofilm formation stimulation at the lowest concentration were selected for further experiments.

2.4 Biofilm formation on polyurethane foam

Flasks containing 1.5 g of polyurethane foam (PUF) rectangular pieces (1 x 1 x 0.5 cm) were filled with 100 ml of corn steep liquor 10% v/v, sterilized and inoculated with 5 ml of a *Pseudomonas monteilii* P26 48 h culture. The culture was static and lasted for 120 h with medium renewal every 48 h. The annonaceous acetogenins itrabin, jetein or an ethanolic extract of *Annona cherimola* were added at the start of the culture and with every medium renewal (at 1.25 ppm for itrabin and jetein and 0.025% for the ethanolic extract). Sterile air was supplied by an air pump through a 0.22 µm nylon filter.

2.5 Biofilm quantification

After the 5 d-incubation, biofilm formation quantification on PUF was carried out according to Quek et al. [24]. Briefly, pieces of PUF from the different immobilization processes were taken, their mass determined, washed twice with 5 ml saline solution (0.9% w/v) to remove the planktonic cells and cut in smaller pieces. The pieces were suspended in 5 ml saline and vortexed for 10 min to detach the immobilized cells. The

CFU/ml of this suspension was determined and the CFU/g_{support} of *P. monteilii* P26 calculated.

2.6 PAH removal

Glass flasks (100 ml) with polypropylene caps containing 5 PUF pieces (sterile or with immobilized cells from each immobilization procedure) and 5 ml of medium JPP supplemented with 50 ppm acenaphthene, fluoranthene and pyrene (Sigma Aldrich, 98%) were incubated in the dark at 30 °C and 180 rpm for 7 days. PAH were added as an acetic solution. Acetone was allowed to evaporate before adding the immobilized cells. After incubation, the PUF was removed and 10 ml acetone was added to the liquid to ensure the complete solubilization of the remaining PAH. A sample of the solution was withdrawn, centrifuged at 6300 g and 25 °C for 10 min and the supernatant was filtered with a 0.2 µm nylon filter. The filtrate was kept at -20 °C until analysis. To determine the PAH concentration sorbed on the support, the PUF pieces were cut in smaller parts and suspended in 5 ml acetone. The suspension was vortexed for 10 min, and then centrifuged and the supernatant filtered and conserved as described above.

Overall PAH removal and PAH bioremoval were calculated as follows:

$$\text{Overall PAH removal (\%)} = 100 \times (C_i - C_f) / C_i$$

PAH bioremoval (%) = $100 / C_i \times [C_i - (C_f + C_s)] - 100 / C_i \times [C_i - (C_{fp} + C_{sp})]$, where C_i is the initial PAH concentration, C_f is the PAH concentration remaining in the liquid at the end of the assay, C_s is the sorbed PAH concentration on PUF-biofilm systems and C_{fp} and C_{sp} are the remaining and sorbed PAH concentration after treatment with sterile PUF respectively.

2.7 PAH quantification

PAH concentration was determined by RP-HPLC applying an external standard calibration curve. The RP-HPLC equipment was an e2695 model from Waters coupled to a PDA detector (Waters 2998, Waters Corporation, MA, USA). The samples were injected in a Phenomenex type spherisorb 5 ODS (2) column (size 250 x 4.60 mm). Two mobile phases were used: 9:1 water:methanol (solvent A) and methanol (solvent B). Each sample run was carried out by the following solvents program: at first, 20% solvent A and 80% solvent B for 10 min, then 10% solvent A and 90% solvent B for 2 min and finally 100% solvent B for 2 min.

2.8 Statistical analysis

One-way analysis of the variance (ANOVA) with Fisher test was performed over the data collected to determine significant differences ($p < 0.05$) between the means obtained from the experiments of PAH removal. Data obtained in the biofilm formation screening and in the biofilm on PUF quantification was analyzed using Mood's Median Test. Obtained p-values lower or equal to 0.05 indicated that two or more medians were significantly different. Medians with overlapping 95% confidence intervals were considered not significantly different. The software used was MINITAB 17 (PA, USA). All experiments were done at least in triplicate and repeated once. A schematic representation of the entire methodology was supplied as supplementary material (fig. S1).

3. Results

3.1 Biofilm formation screening

Biofilm formation stimulation by ACG was evaluated in microplate assays and quantified by crystal violet staining. The effect of the different acetogenins at different concentrations in the biofilm formation of *Pseudomonas monteilii* P26 is shown in table 1. The most significant results were obtained in the presence of jetein (1.25 ppm) and 0.025% extract, in which cases, biofilm formation was stimulated 40% and 75%, respectively. The acetogenins jetein and itrabin and the *Annona cherimola* extract presented the highest absorbance values at the lowest concentration.

3.2 Biofilm on PUF quantification

The biofilm formation on PUF was quantified after the immobilization process by determining the CFU/g_{support} of the immobilized bacteria. Table 2 shows the CFU/g for the different immobilization processes. It was observed that the addition of itrabin and the ethanolic extract produced the highest stimulation. The CFU/g_{support} increased 100-fold in presence of these ACG compared with the biofilm formed in absence of ACG.

3.3 PAH removal

Either PUF with bacteria immobilized on it or sterile were incubated in JPP medium in presence of 50 ppm of acenaphthene, fluoranthene and pyrene for 7 days. After the incubation period, the PUF was removed and the PAH remaining in the liquid and

sorbed to the PUF were determined. The average overall PAH removal by the different systems (sterile PUF, PUF-stimulated biofilm and PUF-unstimulated biofilm) was 99% for acenaphthene, 98% for fluoranthene and 92.5% for pyrene. There was no significant difference between the removal values of the different systems. In average, the removal rate of the different systems was 6.15 ± 0.03 , 6.11 ± 0.09 and 5.75 ± 0.16 ppm/d for acenaphthene, fluoranthene and pyrene respectively. The overall PAH removal and that attributed to biological activity are depicted in figure 2.

Approximately, 15-22% of each PAH was removed by the bacterial biofilms. The sorbed PAH in the sterile PUF system was approx. 80%, while the sorbed PAH in the systems with immobilized cells accounted for the 60% approximately (fig. 3). There was no difference in the amount of sorbed PAH between the different immobilization procedures ($p>0.05$, see table S1 for detailed p-values).

4. Discussion

The biofilm screening experiment on polystyrene microplates allowed selecting the acetogenins itrabin and jetein and the *Annona cherimola* extract for biofilm formation on PUF, for presenting the highest stimulation percentages at the lowest concentration. These ACG also showed strong biofilm stimulation on PUF. To the best of our knowledge, this is the first report in which biofilm formation stimulation by ACG was determined on PUF.

In this study, biofilm formation was stimulated by chemical stress. Parellada et al. [11] suggested that ACG exert stress on the bacterial membrane and a specific interaction between ACG and artificial lipid bilayers was identified by Bombasaro et al. [25] and

Di Toto Blessing et al. [26]. Mangwani et al. [27] reported enhanced biofilm growth of *Pseudomonas mendocina* NR802 using calcium which increased exopolymeric substances (EPS) production. In that study, Ca^{2+} -stimulated biofilm on glass showed a phenanthrene removal of 83% in 7 days while the unstimulated biofilm removed 75% in the same time. Mangwani et al. [28] also stimulated biofilm formation of two marine *Pseudomonas* adding synthetic N-acylhomoserin lactones during the immobilization procedure. In that case, up to 11.6% and 16.5% phenanthrene and pyrene removal improvement was obtained, respectively, over the unstimulated biofilms. In this study, however, no enhanced PAH removal was observed despite the enhanced biofilm formation produced in presence of itrafin or the ethanolic extract of *Annona cherimola*. It is well known that PAH produce hydrophobic damage and, in response, the bacterial cells protect themselves by producing EPS thus forming biofilm [29]. It seems that during the 7 day incubation in the PAH removal assay, the originally non stimulated biofilms reached a similar bacterial number as the previously ACG-stimulated biofilms due to the stress exerted by PAH (increased biofilm thickness was clearly visible after the assay). This would explain the similar values of PAH bioremoval obtained for the different biofilm-PUF systems after 7 days. Probably, the PAH removal rate of stimulated biofilms was higher than unstimulated biofilms due to higher cell number. However, at 7 days the removal values of both systems were already equal.

The sorbed PAH on sterile PUF was 80% while the sorbed PAH in biofilm-PUF systems was 60%. This 20% difference between the abiotic control and the biofilms is the percentage that could be attributed to biodegradation or even bioaccumulation phenomena [30]. Interestingly, according to Isaac et al. [31], *Pseudomonas monteilii* P26 had not been able to remove pyrene at all when using planktonic cells after 21 days

of incubation. The results obtained in this work shows the advantages of using biofilms over free cells for PAH removal as it was already reported by Zhang et al. [29]. PAH sorption phenomena, either on PUF or in EPS, were responsible for the major removal observed. This could be due to the fact that the PAH concentration used was below the sorption capacity of PUF or the biofilm-PUF system. It would be expected that increasing the concentration would saturate the PUF and biodegradation or bioaccumulation would become the major removal processes. Lin et al. [32] illustrated this point when using immobilized cells of *Acinetobacter venetianus* on bagasse for the removal of tetradecane. In that work, there was no difference between the amount of tetradecane removed by bagasse or the immobilized cells at 50 ppm. At 500 ppm, however, the immobilized cells removed significantly more tetradecane than the sterile bagasse. In this work, it was observed that, while PAH removal was similar for the sterile PUF and the biofilm-PUF systems, the amount of sorbed PAH in the latter was less compared to the cell free PUF, probably due to degradation or accumulation of sorbed PAH as explained above. This would allow a major number of reuses of the biofilm-PUF systems than the PUF alone because saturation would not occur as quickly as in the sterile PUF system.

5. Conclusion

Several annonaceous acetogenins (ACG) were purified, identified and tested for its capacity of stimulating biofilm formation on *Pseudomonas monteilii* P26. The ACG with the highest stimulation potential were used in the immobilization of this polycyclic aromatic hydrocarbon (PAH) degrading strain on polyurethane foam (PUF). Strong biofilm formation stimulation was obtained with itrabin and the ethanolic extract of

Annona cherimola, however, this did not enhance PAH removal. Sorption phenomena by the support and the EPS present in biofilms were the major responsible of the PAH removal, although PAH biodegradation or bioaccumulation by the immobilized cells was evidenced. Further experiments will aim to ascertain if the stimulated biofilms are indeed more promising tools for PAH removal than the unstimulated ones. The removal of higher PAH concentrations and the reuse of the immobilized cells will be also tested.

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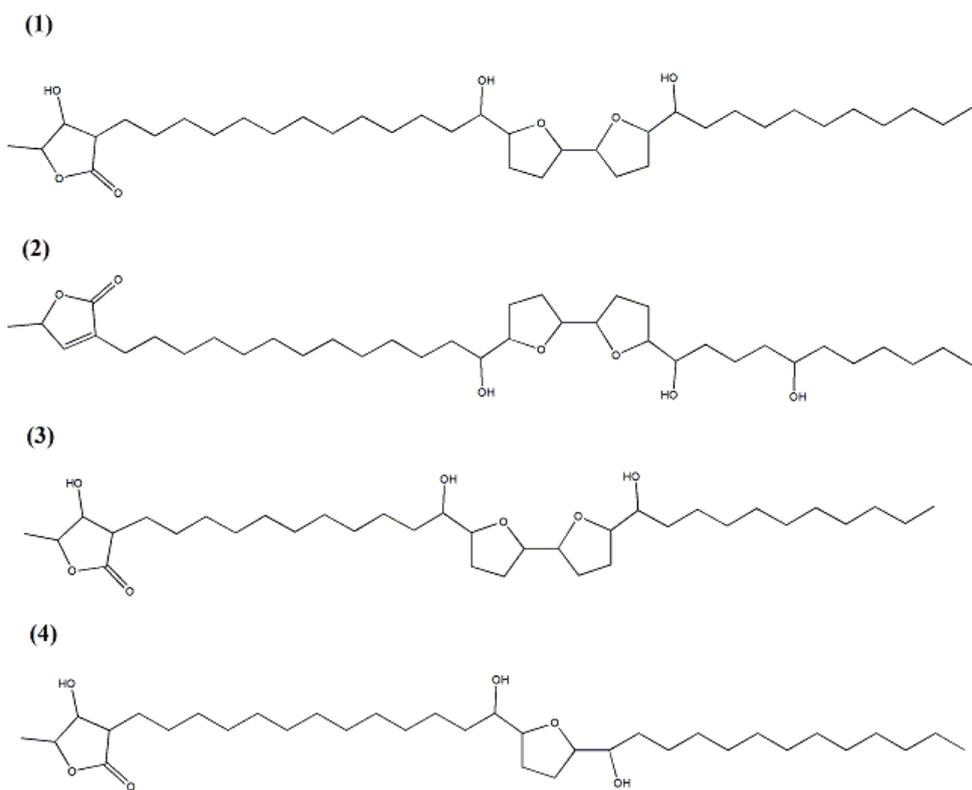


Figure 1: annonaceous acetogenins used in this study. (1) Laherradurin. (2) Squamocin.

(3) Itrabin. (4) Jetein.

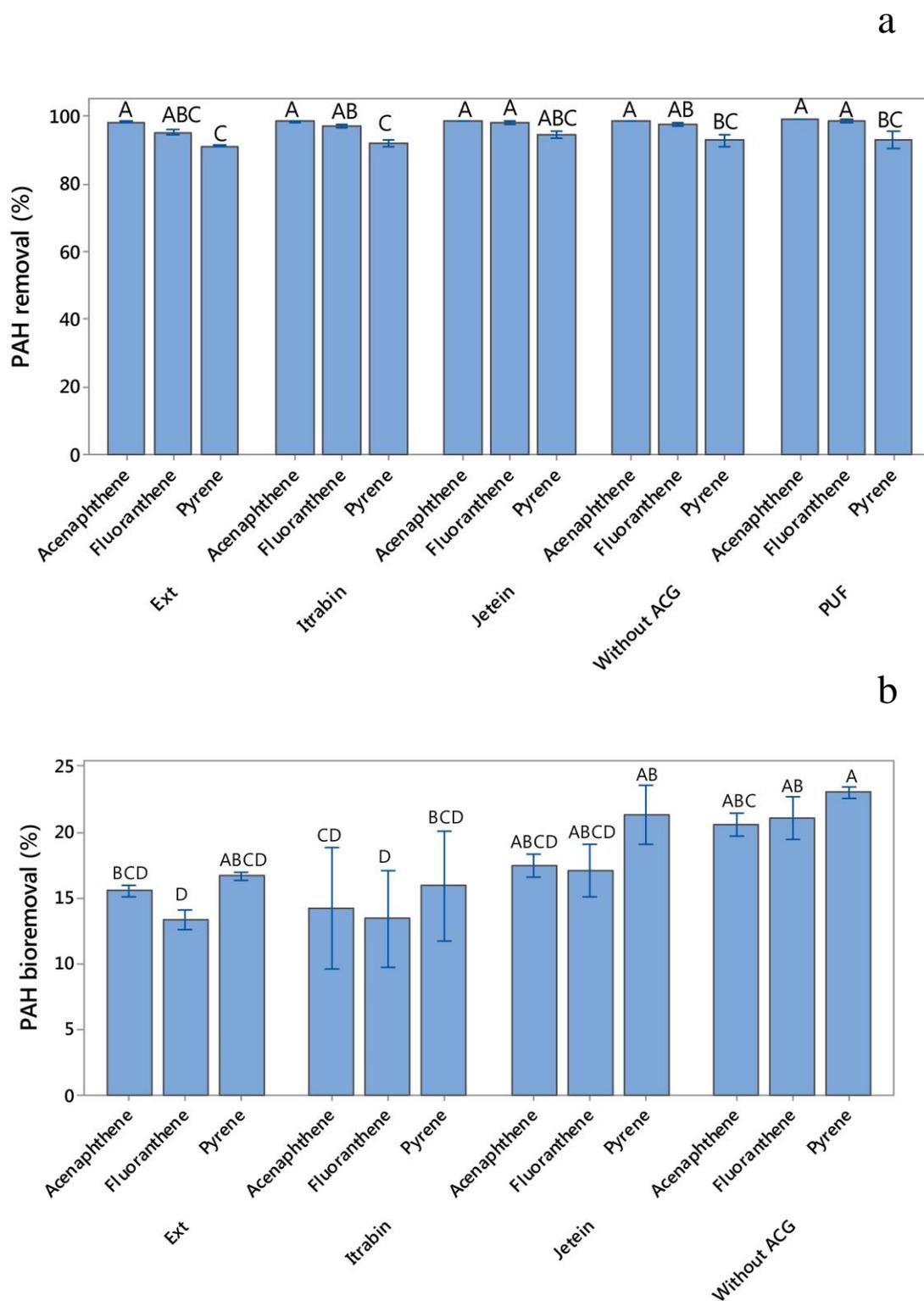


Figure 2: (a) PAH removal percentages for the different systems assayed. PUF: sterile polyurethane foam. Ext, Itrabin, Jetein: biofilms on polyurethane foam formed either in presence of 0.025% *Annona cherimola* pulp ethanolic extract, 1.25 ppm itrabin or 1.25 ppm jetein respectively. Without ACG: biofilm on polyurethane foam formed without

annonaceous acetogenins. (b) PAH bioremoval percentages of the different biofilms on polyurethane foam formed either in presence of 0.025% *Annona cherimola* pulp ethanolic extract (Ext), 1.25 ppm itrabin, 1.25 ppm jetein or without annonaceous acetogenins (without ACG). Data correspond to means of triplicate values and error bars correspond to one standard deviation. Mean values that present the same letter are not statistically different ($p>0.05$).

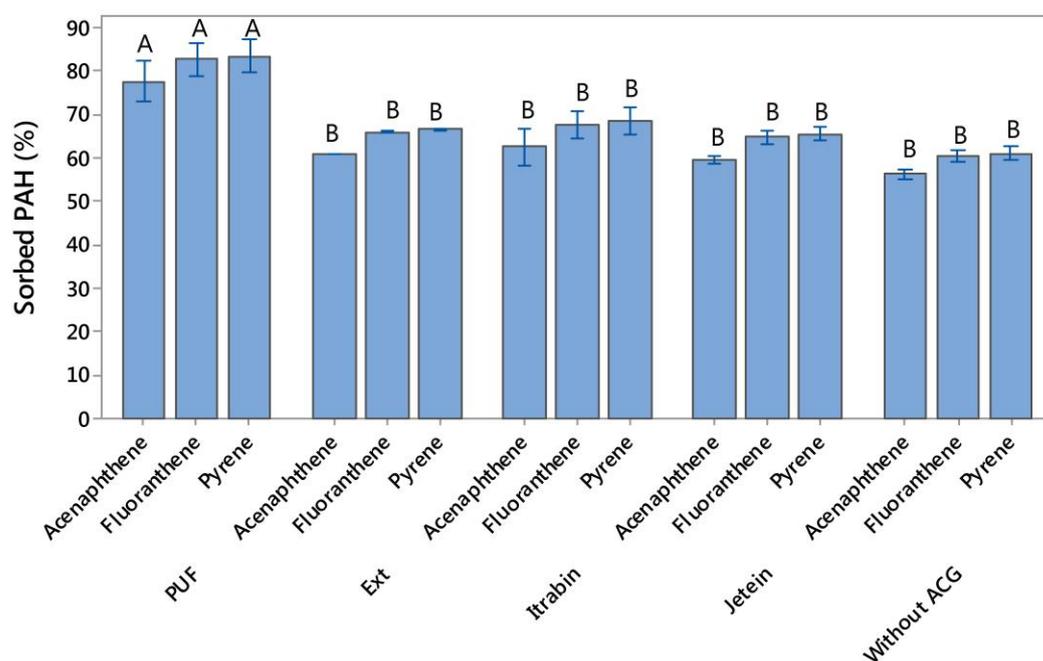


Figure 3: PAH sorption percentages for the different systems assayed. PUF: sterile polyurethane foam. Ext, Itrabin, Jetein: biofilms on polyurethane foam formed either in presence of 0.025% *Annona cherimola* pulp ethanolic extract, 1.25 ppm itrabin or 1.25 ppm jetein respectively. Without ACG: biofilm on polyurethane foam formed without annonaceous acetogenins. Data correspond to means of triplicate values and error bars correspond to one standard deviation. Mean values that present the same letter are not statistically different ($p>0.05$).

Table 1: Screening of biofilm formation stimulation of different annonaceous acetogenins (ACG) and the ethanolic extract of *Annona cherimola* pulp by crystal violet staining assay. Medians sharing a letter are not statistically different.

Condition	Abs 560nm (95% Confidence Interval)
<i>Annona cherimola</i> extract (0.025%)	0.419 (0.297-0.443) ^a
<i>Annona cherimola</i> extract (0.25%)	0.316 (0.258-0.384) ^{abc}
Itrabin (1.25 ppm)	0.296 (0.266-0.337) ^{ab}
Itrabin (2.5 ppm)	0.257 (0.245-0.290) ^{bc}
Jetein (1.25 ppm)	0.338 (0.309-0.369) ^a
Jetein (2.5 ppm)	0.302 (0.271-0.366) ^{ab}
Squamocin (1.25 ppm)	0.255 (0.218-0.309) ^{abc}
Squamocin (2.5 ppm)	0.300 (0.294-0.307) ^a
Laherradurin (1.25 ppm)	0.252 (0.238-0.278) ^{bc}
Laherradurin (2.5 ppm)	0.285 (0.267-0.323) ^{ab}
Without stimulation	0.240 (0.217-0.265) ^c

Table 2: Biofilm on polyurethane foam quantification for the different immobilization conditions. Medians sharing a letter are not statistically different.

Condition	CFU/g x 10E+8 (95% Confidence Interval)
<i>Annona cherimola</i> extract 0.025%	35.19 (5.58-64.80) ^a
Itrabin 1.25 ppm	30.10 (2.24-31.40) ^{ab}
Jetein 1.25 ppm	1.48 (1.10-3.25) ^{bc}
Without stimulation	0.21 (0.21-0.31) ^c